Simultaneous targeting of B-cell malignancies and human immunodeficiency virus with bispecific chimeric antigen receptor T cells

Infection with the human immunodeficiency virus (HIV) confers an increased risk over the general population of developing cancer. More than 40% of people living with HIV (PLWH) develop HIV/acquired immunodeficiency syndrome (AIDS)-associated lymphoma, which is the leading cause of cancer-related mortality in this population.[1] CD19-targeted chimeric antigen receptor (CAR) T-cell therapy has transformed the treatment of relapsed or refractory (r/r) B-cell lymphomas. However, there is little experience using CAR T cells to treat PLWH who have r/r lymphoma due to the historical exclusion of these patients from clinical trials.[2] Recent reports have demonstrated the feasibility of treating PLWH with autologous CD19 CAR T cells for their lymphoma.[2,3] We and others have also developed a CAR T-cell platform that targets HIV-infected cells;[4,5] our anti-HIV CAR uses the single chain variable fragment (scFv) derived from the N6 broadly neutralizing antibody,[6] and has efficacy against HIV both *in vitro* and *in vivo* preclinical studies.[4] Given the feasibility of using CAR T cells in PLWH, we hypothesized that a dual targeting approach could potentially address both lymphoma and HIV infection simultaneously. To that end, we leveraged our humanized CD19 (huCD19) and N6 monotherapy CAR platforms to develop tandem, bispecific N6-huCD19 CAR T cells.[4,7] Here we demonstrate that the N6-huCD19 CAR T cells were highly effective against both B-cell lymphoma and HIV-infected cells, and exhibited sustained efficacy upon rechallenge with either target disease *in vitro*. We determined that it was feasible to generate N6-huCD19 CAR T cells from leukapheresis products from PLWH, which had similar efficacy against both B-cell lymphoma and HIV as N6-huCD19 CAR T cells generated from healthy donors. This therapeutic approach offers significant promise for advancing the treatment of B-cell malignancies in PLWH, while offering a potential dual benefit of simultaneously addressing HIV infection.

We initially developed 3 bispecific CAR constructs by inserting both N6[4] and our humanized CD19[7] scFvs in different orientations into a second-generation CAR construct with a 41BB co-stimulatory domain (Figure 1A). We generated bispecific CAR T cells using our naïve and memory (Tn/mem) platform[7] using blood obtained from healthy donors at the City of Hope Donor Apheresis Center under a protocol approved by our Institutional Review Board. Briefly, we isolated Tn/mem cells from peripheral blood mononuclear cells through autoMACS separation, as previously described,[8] and generated CAR T cells by activating Tn/mem cells with CD3/CD28 Dynabeads and transducing with CAR lentivirus (multiplicity of infection [MOI] = 3). We expanded the resulting CAR T cells in X-VIVO media supplemented with recombinant human (rh)IL-2 (Novartis Pharmaceuticals) and rhIL-15 (CellGenix). To evaluate *in vitro* function, we co-cultured bispecific CAR T cells with either CD19$^+$ Raji lymphoma cells or 8E5 cells that express the HIV envelope glycoprotein gp120 as target cells and measured induction of cytotoxicity by flow cytometry. Only the tandem N6-huCD19 variant killed both target cell lines (Figure 1B). Therefore, we selected the tandem N6-huCD19 CAR as our lead bispecific construct.

Following activation and transduction, we observed similar expansion and comparable phenotype of tandem N6-huCD19 CAR T cells and monospecific N6 or huCD19 CAR T cells generated from the same donor, suggesting that combining the two scFvs did not lead to tonic signaling, exhaustion, or senescence (Figure 1C, D). To compare the *in vitro* function of N6-huCD19 CAR T cells and monospecific CAR T cells, we co-cultured CAR T cells with target cells and evaluated production of interferon-γ (IFNγ) and induction of cytotoxicity by flow cytometry; for the IFNγ assay only, we used HEK cells that express the gp120 precursor glycoprotein, gp160 (HEK-gp160), as target cells. Both monospecific N6 or huCD19 CAR T cells produced INFγ when co-cultured with their respective antigen-positive target cells, while tandem N6-huCD19 CAR T cells produced INFγ against both antigen-positive target cells (Figure 2A). Both monospecific N6 CAR T cells and tandem N6-huCD19 CAR T cells produced less INFγ when co-cultured with target cells than huCD19 CAR T cells, which could suggest different kinetics of targeting a viral antigen (e.g., gp160) *versus* a non-viral antigen, as we had previously observed.[4] Consistent with INFγ production and a previous cytotoxicity assay (Figure 1B), N6-huCD19 CAR T cells eliminated both cell lines at levels comparable to the respective monospecific CAR T cells (Figure 2B). This suggests that combining the two scFvs did not impair the ability of either to engage the respective target antigen.

We subsequently evaluated how N6-huCD19 CAR T cells respond to multiple challenges of a single antigen or alternating antigens in a series of repetitive challenge assays. To explore how N6-huCD19 CAR T cells respond to repetitive antigen challenge, we co-cultured CAR T cells with either Raji or 8E5 cells for 48 hours (hr) followed by a second inoculation with the same target cells. We analyzed the ability of the respective monospecific CAR T cells and N6-huCD19 CAR T cells to induce cytotoxicity of target cells

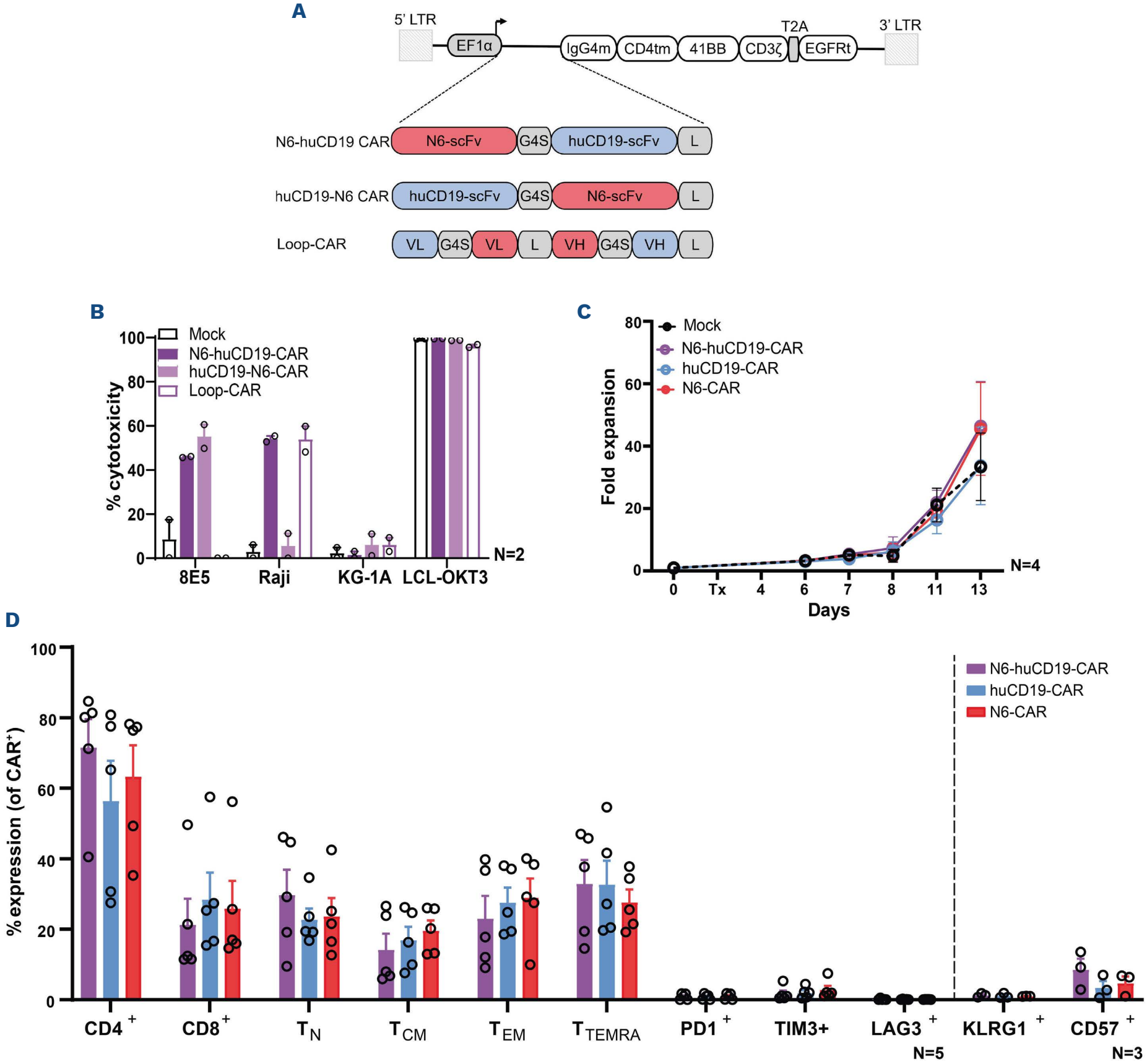

Figure 1. N6-huCD19 tandem chimeric antigen receptor T cells eliminated both lymphoma and human immunodeficiency virus. (A) Bispecific chimeric antigen receptor (CAR) construct designs include tandem CAR (N6-huCD19 and huCD19-N6) and Loop-CAR. For the tandem CAR constructs (N6-huCD19 and huCD19-N6), the scFvs were separated by a G4S linker, and in the loop construct (Loop-CAR), the huCD19 and N6 variable light and heavy chains were separated by G4S and Whitlow linkers (L). Constructs were generated by Gibson cloning of scFvs into a second-generation CAR backbone consisting of IgG4 Fc spacer with 2-point mutations, CD4 transmembrane, 41BB co-stimulatory and CD3ζ signaling domains. (B) Cytotoxicity of bispecific CAR T cells. CAR T cells ($5x10^3$) were co-cultured with GFP^+ target cell lines 8E5, Raji, LCL-OKT3 (positive control), or KG-1A (negative control) at an effector:target ratio of 1:2 for 4 days. The number of remaining viable GFP^+ cells were counted using multicolor flow cytometry. The percentage of cytotoxicity was calculated as 100%-%(remaining tumor cells in CAR T-cell group)/ remaining tumor cells in mock group); N=2 donors. (C) Expansion of monospecific huCD19 or N6 CAR T cells, N6-huCD19 CAR T cells, or mock T cells generated from the same donor; N=4 donors. (D) Phenotypic characterization of indicated CAR T cells on day 13 of culture; N=3-5 donors. Cells were stained with fluorochrome-conjugated monoclonal antibodies against CD3, CD4, CD8, CD62L, CD45RA, CD45RO, CD57 (BD Biosciences), CCR7, EGFR(CAR), KLRG1 (Biolegend), PD1, TIM3 (Invitrogen) and LAG3 (LSBio), and analyzed on a MACSQuant Analyzer 10 (Miltenyi Biotech) after dead cell exclusion with DAPI (Invitrogen).

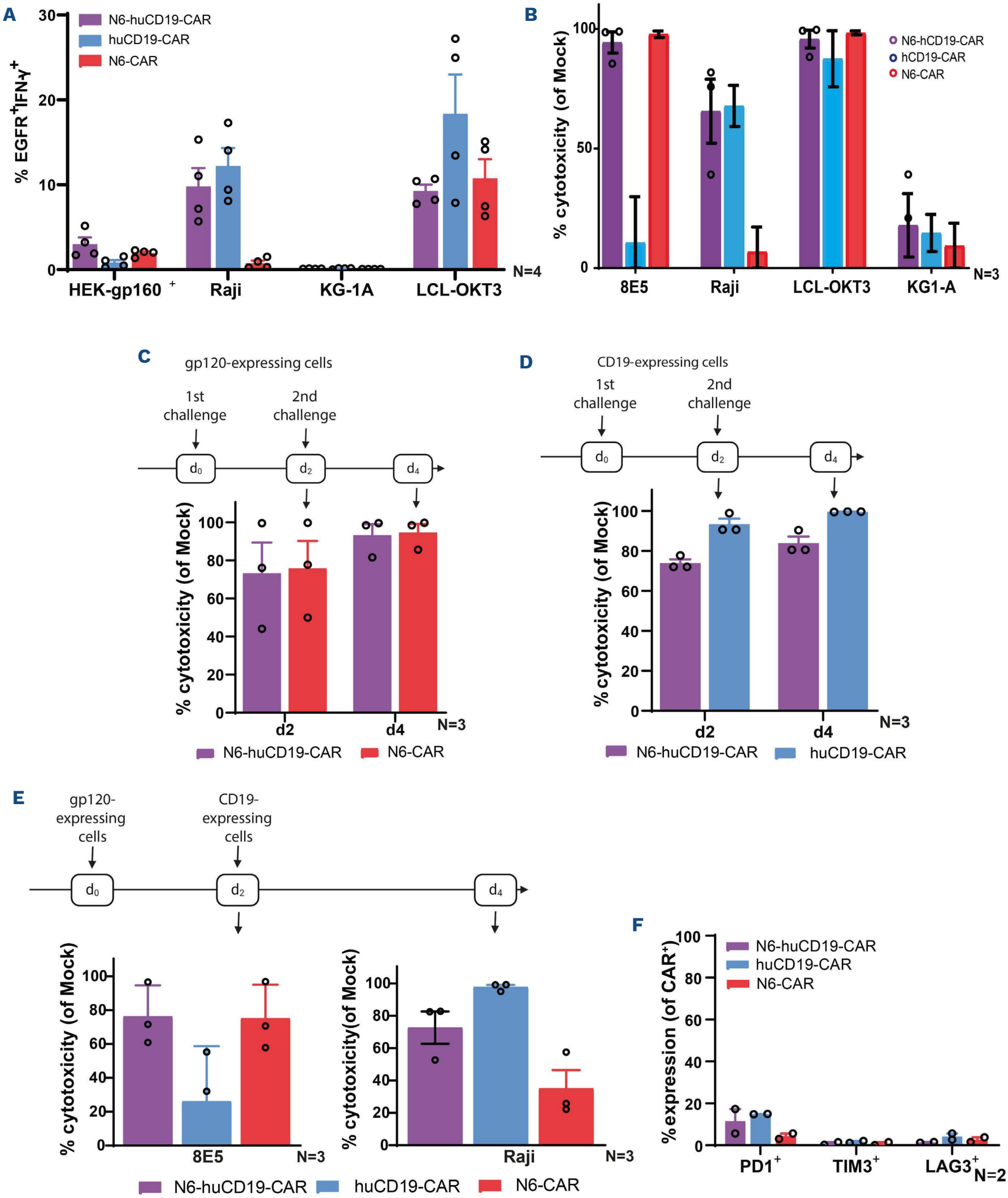

Figure 2. N6 huCD19 chimeric antigen receptor T cells have comparable efficacy to monospecific chimeric antigen receptor T cells. (A) Chimeric antigen receptor (CAR) T cells were activated for 6 hours with indicated target cell lines at an effector-to-target ratio of 1:1 in the presence of Brefeldin A (BD Biosciences). Cells were stained with FVD Viability (Thermo Fisher Scientific), fixed, permeabilized, and stained with intracellular antibody for IFNγ (BD Biosciences). Flow cytometry was performed; N=2 do-

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nors. (B) Cytotoxicity of CAR T cells was evaluated as in Figure 1B; N=3 donors. The capacity of CAR T cells to respond in a repetitive cytotoxicity assay by co-culturing CAR T cells with 8E5 (C) and Raji (D) was evaluated; N=3 donors. (E) The capacity of CAR T cells to respond to alternating antigen challenges was evaluated. CAR T cells were co-cultured for 2 days (d) with 8E5 cells followed by a second challenge with Raji; N=3 donors. (F) Following 4 days of alternative antigen challenges, CAR T cells were analyzed for the indicated exhaustion markers.

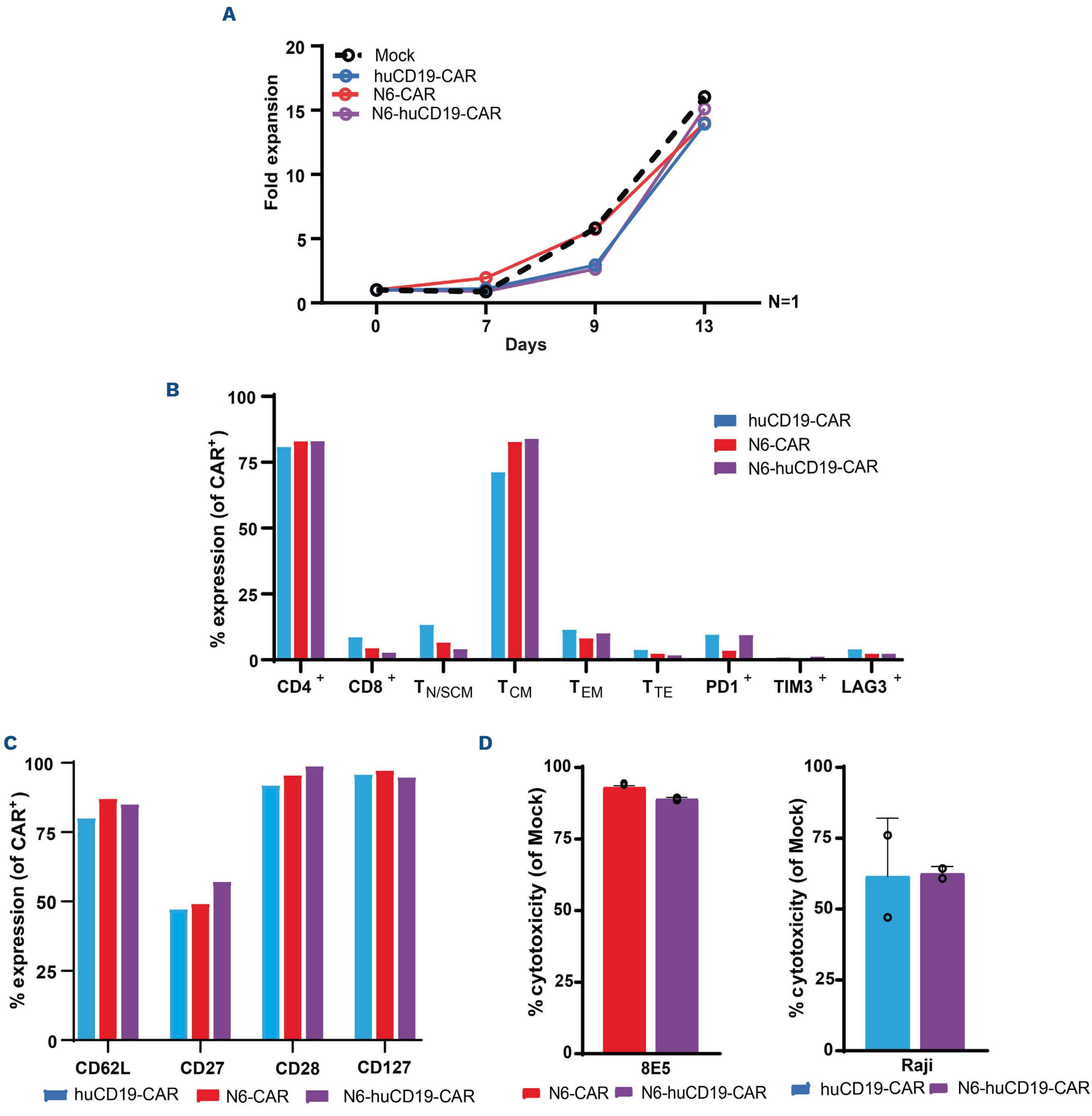

Figure 3. Successful generation of N6-huCD19 chimeric antigen receptor T cells using leukapheresis products from people living with human immunodeficiency virus. (A) Expansion and (B) phenotype of chimeric antigen receptor (CAR) T cells from people living with human immunodeficiency virus (PLWH) donor in the presence of 43 nM darunavir and 279 nM enfuvirtide supplemented twice a week. (C) Comprehensive memory marker analysis of CAR T cells from PLWH donor, including CD62L, CD28, CD27 (BD Biosciences), and CD127 (Biolegend). Cytotoxicity of PLWH-derived CAR T cells against (D) 8E5 and (E) Raji cells.

48 hr following each challenge. N6-huCD19 CAR T cells had similar efficacy to monospecific CAR T cells against their respective antigen positive cells for both the first and second challenge (Figure 2C, D), suggesting that repetitive stimulus did not impact the ability of either monospecific or bispecific CAR T cells to respond to target antigen. However, since it is possible that CAR stimulus through one antigen may impact the ability of bispecific CAR T cells to respond to subsequent second antigen stimulus, we repeated the repetitive antigen challenge and alternated target antigens at rechallenge (i.e., co-culture with 8E5 cells for 48 hr followed by rechallenge with Raji cells). In this repetitive challenge, N6-huCD19 CAR T cells induced cytotoxicity of both 8E5 and Raji cells at similar levels to that induced by the respective monospecific CAR T cells (Figure 2E). Following the repetitive antigen stimulus, the N6-huCD19 CAR T cells did not have increased expression of exhaustion markers over monospecific N6 or huCD19 CAR T cells (Figure 2F).

We had previously generated CAR T cells from PLWH by using the antiretroviral therapy (ART) drugs, darunavir and enfuvirtide, during *ex vivo* culture.[4] Therefore, we used this technique to determine the feasibility of generating functional N6-huCD19 CAR T cells using blood from PLWH (Zen-Bio Inc.). Under ART, mock, monospecific, and bispecific CAR T cells expanded with comparable kinetics over the 13-day culture period (Figure 3A). The resulting CAR T cells also had a high percentage of $CD4^+$ cells and central memory T cells, and low expression of exhaustion markers (Figure 3B). To further evaluate the phenotype of CAR T cells generated from PLWH, we checked expression levels of other memory markers, including CD62L, CD27, CD28, and CD127. The CAR T-cell products generated from the specific PLWH donor expressed high levels of all memory markers and enriched CD4 phenotype (Figure 3C). To determine if N6-huCD19 CAR T cells from PLWH were functional, we co-cultured CAR T cells with 8E5 or Raji cells and measured cytotoxicity. As for CAR T cells generated from healthy donors, we observed comparable functionality of monospecific and N6-huCD19 CAR T cells generated from PLWH (Figure 3D).

Here, we developed a novel bispecific CAR construct that can target both lymphoma and HIV-infected cells. The tandem N6-huCD19 CAR T cells targeted and eliminated both $CD19^+$ and $gp120^+$ cell lines at comparable levels to the respective monospecific CAR T cells (Figure 2). Moreover, N6-huCD19 CAR T cells could eliminate target cells despite repetitive challenge of a single antigen (CD19 or gp120) or alternating between each target antigen (gp120 followed by CD19). Finally, we show that N6-huCD19 CAR T cells could be generated from PLWH, and that they have similar efficacy to those generated from healthy donors. These results demonstrate the potential of developing CAR T cells that target multiple diseases simultaneously.

While this study is focused on HIV and B-cell lymphoma, this platform could potentially be extended to other cancers for which PLWH are at particularly high risk, such as cervical cancer[9] and Kaposi sarcoma.[10] Alternatively, a similar tandem CAR design could be utilized to mitigate opportunistic infections post CAR T-cell therapy which have been observed in approximately 80% of patients treated with CAR T cells, and include cytomegalovirus (CMV) and fungal infections.[11,12] To combat CMV, we previously reported on isolating CMV-specific T cells to make bispecific CAR T-cell product that could target a B-cell malignancy as well as CMV infection, and are currently testing its efficacy clinically.[4,13] Other groups have described CAR designs to target fungal infection.[14,15] While these strategies may alleviate the burden of infections post CAR T-cell infusion, the isolation of virus-specific T cells or developing a second monospecific CAR construct adds to the cost and complexity of the final CAR T-cell product. Therefore, developing a single CAR construct that targets multiple diseases offers new potential to tailor CAR T-cell therapy to medically complex patients.

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Disclosures

No conflicts of interest to disclose.

Contributions

XW, SJF, RN, JHB and JAZ played a role in conceiving the concept.

XW supervised the study. RU and SP designed and performed experiments, and analyzed the data. BG conducted experiments. SP, RU, XW and MCC wrote the manuscript.

Data-sharing statement

The data generated in this study are available upon request from the corresponding author.

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